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RESEARCH ARTICLE

Multi-strain co-cultures surpass blends for broad spectrum biological control of maladies of potatoes in storage[†]

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Pseudomonas fluorescens strains S11:P:12, P22:Y:05, and S22:T:04 and *Enterobacter cloacae* strain S11:T:07 have been documented to suppress four important storage potato maladies – dry rot, late blight, pink rot, and sprouting. This research investigates the efficacy and consistency of strain mixtures produced by co-culturing strains together in one vessel or by blending them together after separate cultivations in pure cultures. Pure and co-cultures were produced in flask or fermentor cultures, viable cell concentrations were assessed using a nutrient-based selective plating method to identify and enumerate strains, and the efficacy of treatments was assessed with respect to dry rot, pink rot, late blight or sprout suppression. Experiments were designed to analyze dry rot suppression versus all strain combinations and the combination method (co-culture or blend). Results of a two-way analysis of variance of disease with strain composition and combination method showed that significantly better dry rot suppression was obtained by co-cultures ($30.3 \pm 2.4\%$ relative disease) than by similar strain blends of pure cultures ($41.3 \pm 2.4\%$) ($P < 0.001$). During a 3-year study, both biocontrol efficacy and consistency were assessed in 16 laboratory and small pilot trials simulating commercial storages. Three-strain co-culture had a lower mean disease rating than the blend in 9 of 16 experiments examining control of the three diseases and sprouting. The co-culture led other treatments in incidences of significant malady reduction relative to the control: 14 of 16 attempts for co-culture, 11 of 16 attempts for blend, 10 of 13 attempts for pure S11:P:12, 8 of 13 attempts for S22:T:04, and 9 of 13 attempts for P22:Y:05. Using relative performance indices to rank treatment performance across all experiments, the co-culture treatment ranked significantly higher than the blend. A synergy analysis suggested that co-culturing strains stimulated inter-strain activities to boost biocontrol efficacy and consistency, a feature not developed in strains grown separately and mixed just prior to addition to potatoes.

Keywords: *Solanum tuberosum*; gram-negative bacteria; *Pseudomonas fluorescens*; *Enterobacter cloacae*; biological control; sprout inhibitor; biological fungicide; late blight; dry rot; pink rot; co-culture; mixed culture; mixed strain

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Introduction

Eighteen Gram-negative bacteria were originally discovered and developed as biocontrol agents to protect potatoes entering storage from Fusarium dry rot incited by *Gibberella pulicaris* (Schisler and Slininger 1994; Slininger, Schisler, and Bothast 1994; Slininger, Schisler, and Bothast 1996a; Schisler, Slininger, Bothast 1997; Schisler, Bothast, and Slininger 1998), and they significantly reduced the level of dry rot disease in pilot trials (Schisler, Slininger, Kleinkopf, Bothast, and Ostrowski 2000). Top dry rot suppressive strains include *Pseudomonas fluorescens* biovar 5 (S11:P:12 NRRL B-21133 and P22:Y:05 NRRL B-21053), *Pseudomonas fluorescens* biovar 1 (S22:T:04 NRRL B-21102) and *Enterobacter cloacae* (S11:T:07 NRRL B-21050). All of these strains have been documented to suppress sprouting (Slininger, Burkhead, Schisler, and Bothast 2000; Slininger, Schisler, Burkhead, and Bothast 2003), with *Pseudomonas fluorescens* S11:P:12 (NRRL B-21133) being the most effective in this novel ability, and *E. cloacae* S11:T:07 ranking second. In addition, the strains have been shown to suppress late blight incited by *Phytophthora infestans* US-8 mating type A2 in laboratory bioassays and small pilot simulations of commercial storage conditions with top performance shown by the following treatments: a mixture of four strains (comprised of S11:P:12 + P22:Y:05 + S22:T:04 + S11:T:07) > strain S22:T:04 used alone > strain S11:P:12 used alone (Slininger et al. 2007). Most recently, we showed the ability of several of our dry rot antagonistic bacteria to suppress pink rot disease incited by *Phytophthora erythroseptica*, including S11:T:07 which ranked first and S22:T:04 which was third (Schisler et al. 2009).

The present research further explores the level and consistency of pest control achievable with these beneficial bacteria when they are either grown together in a co-culture or grown separately in pure cultures and then applied individually or mixed to form a blend of the strain populations. Treatments applied in both laboratory wounded potato bioassays and small pilot trials simulating commercial storage conditions are tested, as well as treatments challenged with dry rot, late blight, pink rot and sprouting. Consistence of biocontrol efficacy and broad pest coverage are both major factors influencing the economics of a successful product that are addressed in this research. Several researchers have reported that mixtures of strains can enhance and/or improve the consistency of biological control (among these, Pierson and Weller 1994; Duffy and Weller 1995; Duffy, Simon, and Weller 1996; Janisiewicz 1996; Leeman et al. 1996; de Boer, van der Sluis, van Loon, and Bakker 1999; Guetsky, Shtienberg, Elad, and Dinoor 2001; Krauss and Soberanis 2001; Hwang and Benson 2002; Schisler, Boehm, and Slininger 2005; Cruz, Gaitan, and Gongora 2006). Our preliminary research has shown that formulations containing multiple strains of our dry rot antagonists performed more consistently than individual strains (Schisler et al. 1997; Slininger et al. 2007), including when subjected to 32 storage environments varying in potato cultivar, harvest year, potato washing procedure (microflora exposure), temperature, and storage time (Slininger, Schisler, and Kleinkopf 2001). Despite the apparent advantages of applying strain mixes, the disadvantages for the manufacturer are capital costs, operation, maintenance, registration and management of a different fermentation for each strain used in a mix. A potential way around this obstacle is to co-culture the strains together in one fermentor. However, this avenue has not previously been reported in the literature on biological control.

Materials and methods

Bacterial antagonists

Suppressive strains *Pseudomonas fluorescens* biovar 5 (S11:P:12 NRRL B-21133 and P22:Y:05 NRRL B-21053), *Pseudomonas fluorescens* biovar 1 (S22:T:04 NRRL B-21102) and *Enterobacter cloacae* (S11:T:07 NRRL B-21050) isolated by Schisler and Slininger (1994) were stored lyophilized in the ARS Patent Culture Collection (NCAUR, USDA, Peoria, IL). Stock cultures of bacteria in 10% glycerol were stored at –80°C. Glycerol stocks were streaked to 1/5 strength trypticase soy broth agar plates (1/5 TSA; Difco Laboratories, Detroit, MI) which were incubated for 2–3 days at 25°C and refrigerated for up to 1 week as a source of preculture inoculum.

Cultivation medium

SDCL medium was prepared with 2 g/L each K_2HPO_4 and KH_2PO_4; minerals including 0.1 g/L $MgSO_4(7H_2O)$, 10 mg/L NaCl, 10 mg/L $FeSO_4(7H_2O)$, 4.4 mg/L $ZnSO_4(7H_2O)$, 11 mg/L $CaCl_2(2H_2O)$, 10 mg/L $MnCl_2(4H_2O)$, 2 mg/L $(NH_4)_6Mo_7O_{24}(4H_2O)$, 2.4 mg/L H_3BO_3, 50 mg/L EDTA; 0.01 g/L each of growth factors adenine, cytosine, guanine, uracil, thymine; 0.5 mg/L each of vitamins thiamine, riboflavin, calcium pantothenate, niacin, pyridoxamine, thioctic acid; 0.05 mg/L each of vitamins folic acid, biotin, B_{12}; 15 g/L Difco vitamin-free casamino acids, 0.15 g/L tryptophan, 0.6 g/L cysteine, and 15 g/L glucose. Macro minerals, amino acids, glucose, and acidified purines and pyrimidines were autoclaved separately. Vitamins and trace minerals <0.1 g/L were filter sterilized. After combining sterilized ingredient groups, pH was adjusted to 7 with NaOH.

Shake-flask cultivations of bacterial inocula for test cultures

Fifty-mL precultures were the source of inocula for fermentors and shake-flask test cultures. Pre-cultures contained SDCL medium in 125-mL flasks, and were shaken at 250 rpm (2.5 cm eccentricity) and 25°C in a New Brunswick Psychrotherm incubator. Cultures harvested after 24 h incubation were used to supply bacteria for baffled flask or fermentor test culture inoculations. Typical cell accumulations reached $\sim 0.5–1 \times 10^{10}$ per mL.

Baffled shake-flask cultivations of bacteria

Test cultures of 75-mL volume were incubated in 500-mL baffled flasks shaken at 25°C and 250 rpm. The culture medium was the SDCL medium above enriched to contain 40 g/L glucose, 60 g/L casamino acids, 0.6 g/L tryptophan, and 2.4 g/L cysteine. Bacteria were inoculated to an initial absorbance at 620 nm of 0.1 (1×10^8 viable cells/mL) and harvested after 72 h of incubation. In flask co-cultures, the initial bacterial concentrations were set as such so that each population initially contributed equally to the overall culture absorbance, except that the *E. cloacae* S11:T:07 population in co-cultures was always set at 0.0001 since this strain could exclude the other three *P. fluorescens* strains if inoculated to higher levels.

Fermentor cultivations of bacteria

Bacteria were cultivated in 2-L B. Braun Biostat E or ED fermentors charged with 1.6 L of the SDCL medium above enriched to contain 40 g/L glucose, 60 g/L casamino acids, 0.6 g/L tryptophan, and 2.4 g/L cysteine. Fermentors were controlled at 25°C, pH 7 (with 6*N* NaOH or 3 *N* HCl additives), 1 L/min aeration, and variable stirring 300–1500 rpm to maintain dissolved oxygen at 30% of saturation. To control foaming, a 20% solution of Cognis FBA 3107 was dosed as needed. Bacteria were inoculated to an absorbance at 620 nm of 0.1 (1×10^8 viable cells/mL) and harvested after growth 72 h; at which time, viable cell accumulations were typically $\sim 2–3 \times 10^{10}$ per mL, giving an absorbance of ~ 20. Fermentor co-cultures of *Pseudomonas fluorescens* strains were inoculated to have the following initial absorbances, unless otherwise specified: 0.05 for each of S22:T:04 and S11:P:12 and 0.01 for P22:Y:05.

Enumeration of mixed bacteria populations using selective plating media

Total bacterial biomass (b) was assessed using absorbance at A_{620} where $b = kA$ and $k = 0.408$ g/L per absorbance unit assuming a 1 cm path length (Slininger and Jackson 1992). The viable cell concentrations of each population in mixed cultures or blends were assessed by dilution plating cultures in duplicate to each of three selective agar media: King's Medium B (KMB), 1/5 TSA plus Tetrazolium (TSA-T), and Minimal Medium with Histidine and Tetrazolium (MMHT). Total counts of all strains as well as S11:P:12 were obtained from TSA-T spread plates after 24–48 h incubation at 25°C. Strain S11:P:12 formed a distinctively large diameter diffuse shiny colony becoming creamy with pink concentric rings (2–5 mm). Other strains formed smaller dense round colonies (1–2 mm) with red centers and white borders. A selective count of P22:Y:05 as deep red colonies, white edges (1mm) was obtained from MMHT spread plates after incubation for 48–72 h, while colonies of the other strains used in the study remained relatively tiny and white. The plating tool for P22:Y:05 was developed using Biolog GN plates to screen carbon sources used by the four bacteria, allowing discovery of histidine as a selective carbon source. A specific count of *Enterobacter cloacae* S11:T:07 was determined by picking 30 random non-S11:P:12 colonies from each countable TSA-T plate to KMB plates where the percentage of non-fluorescing colonies could be evaluated. Non-fluorescent colonies on KMB were *E. cloacae*, whereas fluorescent colonies were either *P. fluorescens* P22:Y:05 or S22:T:04.

Plating medium ingredients in the 1/5 TSA-T were: 6 g/L Difco Bacto Tryptic Soy Broth, 15 g/L Difco Bacto Agar, and 0.05g/L 2,3,5-triphenyl-tetrazolium chloride (tetrazolium red, T-8877, Sigma). The medium was prepared by mixing the TSB and agar in distilled water, autoclaving, then mixing in 10 mL of tetrazolium filter-sterilized concentrate to the ~ 60°C agar mix. Solidified plates were refrigerated to preserve the tetrazolium red.

Ingredients in the MMHT were the following: 2 g/L K_2HPO_4, 2 g/L KH_2PO_4, 0.01 g/L $FeSO_4(7H_2O)$, 0.1 g/L MgSO4($7H_2O$), 0.01 g/L NaCl, 0.0044 g/L $ZnSO_4$ ($7H_2O$), 0.011 g/L $CaCl_2$ ($2H_2O$), 0.01 g/L $MnCl_2(4H_2O)$, 0.002 g/L $(NH_4)_6$ $MO_7O_{24}(4H_2O)$, 0.024 g/L H_3BO_3, 0.05 g/L EDTA, 1.26 g/L urea, 5 g/L histidine, 15 g/L BD Difco agar, and 0.05 g/L 2,3,5-triphenyl-tetrazolium chloride. The MMHT was prepared by mixing warm, double-strength autoclaved agar with the

double-strength filter-sterilized nutrient solution and tetrazolium concentrate in a sterile bottle with stir bar. The MMHT nutrient solution preparation was done similarly to the SDCL fermentation medium described above and adjusted to pH 7 before mixing with the agar solution. The medium was poured to plates immediately upon mixing, and the solidified plates, as for TSA-T, were stored refrigerated.

Ingredients in King's Medium B were: 10 g/L BD Difco Bacto Proteose Peptone#3, 10 g/L glycerol, 1.5 g/L K_2HPO_4, 1.5 g/L $MgSO_4(7H_2O)$, and 15 g/L BD Difco Bacto Agar. KMB ingredients were mixed, pH adjusted to 7.2, autoclaved and then molten agar was poured to plates.

Peoria wounded potato bioassay of Fusarium dry rot or pink rot suppression

The wounded potato bioassay of treatment efficacy against *Gibberella pulicaris* (Fr.:Fr.) Sacc. (anamorph: *Fusarium sambucinum* Fuckel) strain R-6380 was originally described in Schisler and Slininger (1994). In the present study, the bacteria treatments were diluted by mixing 0.5 mL culture with 17.5 mL of chilled buffer, and then 1:1 (v/v) with *G. pulicaris* R-6380 at either 1 or 3×10^6 conidia/mL (by hemacytometer count), pending virulence in prior assays. Potato wounds made with a 2mm diameter $\times$ 2 mm length steel pin were thus co-inoculated with treatment and pathogen by pipetting 5 μL of the 1:1 (v/v) treatment:pathogen mixture to each wound. Each bacteria treatment was repeated on six size B Russet Burbank seed potatoes (Wisconsin Seed Potato Certification Program, University of Wisconsin Madison, Antigo, WI) that had been washed and dried a day ahead at room temperature, following prior storage in a cold room $\sim 4^\circ$C. Each potato had four wounds equally spaced around the middle – three wounds receiving bacteria and pathogen and one control wound receiving only pathogen mixed with buffer. Each potato was placed in a plastic weigh boat on a dry 2.54 cm-cut square of Wyp-all L40 all purpose wiper paper towel (Kimberly-Clark Worldwide, Inc.). Boats were held in trays that were supplied two dry Wyp-alls over the top of potatoes and two Wyp-alls wet with 40 mL of water each and placed on either side of the tray, plastic bagged, and stored 21 days at $>90\%$ relative humidity and 15°C. After storage each potato was quartered, slicing through the center of each of the four wounds. The extent of disease in each wound was rated by adding the greatest depth and width measurements (mm) of discolored necrotic tissue extending below and to the sides of the wound. Relative disease (%) was calculated as $100\times$(wound disease rating/average disease rating of wounds receiving pathogen only). Similarly, pink rot suppression was assayed on wounded potatoes. Zoospores of the causative pathogen *Phytophthora erythroseptica* strain 02-25 were produced per Schisler et al. (2009) and suspended at 3×10^4 zoospores/mL buffer before mixing 1:1 with biocontrol agent treatments (or buffer control). Then 5 μL of each treatment-pathogen mixture was applied to 10 replicate wounds, each wound on a different potato. Each potato had two wounds and was used to test two treatments. Tubers were stored as for the dry rot assay, and pink rot development was rated after 1 week, using the same lesion width plus depth method as described above for dry rot.

Kimberly small pilot evaluations of biocontrol efficacy – late blight, pink rot, sprouting

Late blight

Phytophthora infestans JMUIK-2002 (US-8, mating type A2) was obtained from Dr Jeff Miller (University of Idaho, Aberdeen, ID) for small pilot testing of disease suppression under commercial storage conditions at the University of Idaho Kimberly Research and Extension Center, Kimberly, ID. Kimberly rye agar plates were grown in darkness for 2 weeks at 18°C, where rye agar was prepared as follows: 60 g rye seed were soaked over night; seeds were then ground in a blender for 3 min and strained out using four layers cheesecloth; deionized water was poured through pulp until 1 L total volume suspension was collected; 10 g glucose and 15 g agar were added just prior to autoclaving. Plates were harvested by adding 10 mL of ~4°C water per plate and scraping with a glass hockey stick to recover about 9 mL containing 2×10^5 sporangia/mL. For tuber inoculation, 2.5 L of 4×10^4 sporangia/mL were chilled for 1.5 h and then warmed to room temperature ~15–18°C for 45 min to liberate zoospores.

The suspension of *P. infestans* was sprayed onto all of the tubers for the trial at a rate of 1.6 mL per 8 oz. tuber (0.8 mL/tuber first to one side plus 0.8 mL/tuber to the other side). An air-assist syringe sprayer was used with a Delevan brass nozzle (#4, 80°LF). The sprayer and nozzle were sterilized between treatments by rinsing once with 70% ethanol and then three times with distilled water. Potatoes were sprayed in groups of about one hundred spread out on plastic sheeting which could be drawn up to cover the potatoes to retain moisture until the bacterial treatments or water controls were applied. Pathogen-inoculated potatoes were collected into three replicate groups, and allowed to set no more than 45 min prior to treatment with biocontrol agents. Bacterial cultures were harvested from fermentors and stored refrigerated or in chilled shipping coolers 2–5 days before the trial. On the day of the trial, cultures were diluted in half with cold distilled water and returned to the refrigerator until application. Each bacterial treatment or water control treatment was then similarly sprayed at a rate of 0.8 mL per tuber to 30 potatoes from each of the three pathogen-treated replicate groups.

Each treatment replicate of 30 tubers was placed in an unventilated plastic box with lid and positioned randomly on shelves in the storage bay which was maintained at 7.2°C (45°F) and 95% relative humidity. After 3 weeks storage, potatoes were peeled and rated based on the percentage of surface area showing the discoloration typical of late blight infection.

Pink rot

Phytophthora erythroseptica strain 01-21, the causative pathogen of pink rot disease, was produced by Mr Shane Clayson and Dr Jeff Miller (University of Idaho, Aberdeen, ID) (Schisler et al. 2009). To release zoospores, plates were chilled on ice for one hour and then allowed to warm to room temperature for 2 h. To prepare pathogen inoculum to be used in each trial, around 50 plates of *P. erythroseptica* (*Pe*) were harvested at a rate of about 5×10^5 zoospores/10 mL distilled water per plate.

Russet Burbank tubers were bruised by tumbling about 75 tubers at a time for 2 min in a padded plastic cement mixer. Enough tubers were bruised to

accommodate four 15-tuber replicates per each treatment. *Pe* suspension (10^4–10^5 zoospores/mL pending virulence) was sprayed onto tubers at a rate of 1.6 mL per 8 oz Russet Burbank or Russet Norkotah tuber. Seventy-five tubers at a time were placed on a plastic sheet-covered spray table, and 60 mL were applied to the first side of the potatoes. Tubers were flipped and 60 mL more were applied to the other side, for a total of 120 mL per 75 tubers, or 1.6 mL per tuber. Tubers were then taken off the table using the plastic sheet, set on the cement floor (~60–65°F) and the edges pulled up to completely cover the tubers and retain moisture. This process was repeated to prepare four pools of bruised, pathogen treated tubers, such that one 15-tuber replicate of each treatment was drawn from each of the four pools. Before experimental bacterial treatments were applied, tubers were left for approximately 15 min after the final replicate of each cultivar was treated with pathogen. This process was then repeated in its entirety for the Russet Norkotah tubers after bacterial inoculation of the Russet Burbank cultivar was complete.

Bacterial treatments and water controls were applied at a rate of 0.8 mL per tuber. As for the late blight evaluation, ready-to-spray bacterial treatments were prepared and set aside in the refrigerator for plating at the end of the day. To form each treatment replicate, 15 tubers from each of the four plastic sheets on the floor were placed onto the spray table, marking the replicate origin. To one side of the 60 potatoes, 24 mL were applied to the 60 tubers, and then another 24 mL were applied after the tubers were flipped. Tubers were then placed into their respective replicate bags at 15 tubers per bag and then all 4 replicate bags placed in the appropriate box. Boxes were then moved immediately into the storage bay and a lid placed on the box. No ventilation was connected to the boxes. The process was then repeated with Russet Norkotah being treated with pathogen, 15 min wait, then bacterial treatments.

Treatment boxes were stored at 17°C for 2 weeks to allow disease development. At the end of the storage period, tubers were sliced longitudinally, allowed to sit at ambient temperature for 30 min and then evaluated for incidence and severity of infection. Severity is defined as the percentage of tuber tissue showing typical disease symptoms.

Sprouting

Biological control treatments and water only control were sprayed at 0.8 mL per 8 oz Russet Burbank potato. For each treatment, three replicate groups of 70 potatoes were sprayed. Each replicate was spread over a plastic sheet-covered table and sprayed with half the volume on one side, flipped, and then the other half of the volume was sprayed to the other side. Each replicate was transferred to a plastic box labeled with treatment and replicate numbers. The plastic sheet was toweled off to minimize excess collection of treatment on the plastic between spraying each replicate and disposed after each treatment was finished. Each box of potatoes was placed randomly in the storage bay, which was held at 45°F and 95% relative humidity with 1 cfm/cwt air circulation. Ready-to-spray treatment samples were refrigerated until plating after finishing all treatment applications. Treatments were monitored by drawing 20 tubers per replicate box and measuring the longest sprout

per tuber, ten-potato total sprout length and ten-potato total sprout weight per replicate sampled. Potatoes were stored in November and finally monitored in April.

Statistical analysis

Analysis of variance (ANOVA) was performed using Sigmastat 2.03 (SPSS, Inc.) or Statistix 8 (Analytical Software) to determine significant main effects and interactions of the variables tested. Pair-wise comparisons were made using Student Newman Keuls (SNK), Least Significant Difference (LSD), or paired *t*-test analysis. The significance criterion applied was generally $P < 0.05$, or otherwise noted in the 0.1–0.001 range.

Results

In both laboratory and small pilot testing of bioefficacy, co-cultured microbial biocontrol strains consistently outperformed pure stains and blends of strains produced individually in pure cultures.

Co-culture versus blend efficacy for all combinations of four strains grown in baffled flasks

In this experiment design all 4-, 3-, and 2-strain combinations as well as pure strains S11:P:12, S22:T:04, P22:Y:05, and S11:T:07 were inoculated to baffled flask cultures. The co-culture and pure culture populations harvested after 72 h had the strain compositions and absorbances listed in Table 1. Pure strain cultures were blended in equal volume to form the 'blend' compositions listed. These treatments were tested in two dry rot wounded potato bioassay experiments, one at 0.5×10^6 *F. sambucinum* conidia/mL and another experiment at 1.5×10^6 conidia/mL per 5 μL wound. The impact of strain composition versus combination method on Fusarium dry rot disease development (percent relative disease) was tested and analyzed using two-way analysis of variance.

Selective plating media not requiring antibiotic markers were designed and used since preliminary experiments indicated that marked strains had the complications of growth rates and potentially metabolisms being different from parent strains, and phenotype drift occurring in the absence of antibiotic pressure. A nutrient-based selective plating scheme was chosen to monitor the individual strain populations in co-cultures, allowing results directly representing the natural biocontrol strains and their kinetic characteristics.

Disease ratings observed in wounded potato bioassays indicated that all biocontrol agent treatments, including co-cultures, blends, and pure cultures significantly reduced dry rot disease development relative to both low and high level pathogen challenges ($P < 0.001$). Since the treatment × pathogen level interaction was not significant, the two pathogen level data sets were combined for further analysis. Subsequent two-way analysis of variance of relative disease with strain composition of treatments by the method of combining strains (blend versus co-culture) showed that significantly better Fusarium dry rot suppression was obtained by co-cultures than by similar strain mixtures created by blending pure strain cultures ($P < 0.001$) (Table 2). The mean relative disease rating ($\pm$ standard error) for

Table 1. One-way analysis of variance of actual and calculated relative disease ratings indicate that strains grown in co-cultures develop synergistic activities that support superior disease suppressiveness compared with blends of pure strains cultivated separately.

	72-h Culture viable cell populations $\times 10^{-10}$ (cells/mL)[e]						
Strain combination method	S11:P:12	S22:T:04	P22:Y:05	S11:T:07	72-h Culture absorbance (620 nm)[a]	Actual relative disease rating[a,b,c] (%)	Calculated relative disease rating[a,b] (%)
Co-culture	0.25	0.0018	0.10	2.4	15.2	28.2	46.2
	0.40	0.56	1.10	0	16.4	39.9	35.9
	0.027	0	0.046	1.1	14.7	39.9	46.5
	0.16	0.0055	0	2.2	16.3	30.7	46.8
	0	0.038	0.10	1.8	16.6	22.5	46.1
	0.44	0	0.57	0	16.3	25.0	37.8
	0.29	1.10	0	0	17.4	26.7	37.3
	0.0031	0	0	0.23	17.3	36.9	47.0
	0	0.42	0.56	0	14.4	23.5	34.2
	0	0	0.017	2.5	15.5	27.1	47.0
	0	0.012	0	2.2	15.2	33.1	47.0
Means					**15.9 ± 1.0 A**	**30.3 ± 6.3 A,a**	**42.9 ± 5.3 A,b**
Blend[d]	0.28	0.40	0.085	0.20		25.4	40.2
	0.37	0.53	0.11	0		30.8	38.4
	0.37	0	0.11	0.27		50.5	43.4
	0.37	0.53	0	0.27		34.4	40.9
	0	0.53	0.11	0.27		45.5	38.6
	0.55	0	0.17	0.00		44.9	41.4
	0.55	0.80	0	0		30.0	39.0
	0.55	0	0	0.40		32.2	45.3
	0	0.80	0.17	0		48.0	35.2
	0	0	0.17	0.40		40.6	42.9
	0	0.80	0	0.40		71.9	39.4

Table 1. (*Continued*).

Strain combination method	72-h Culture viable cell populations $\times 10^{-10}$ (cells/mL)[e]				72-h Culture absorbance (620 nm)[a]	Actual relative disease rating[a,b,c] (%)	Calculated relative disease rating[a,b] (%)
	S11:P:12	S22:T:04	P22:Y:05	S11:T:07			
Means						**41.3 ± 13.1 B,a**	**40.4 ± 2.8 A,a**
Pure	1.1				16.4	44.0	44.0
		1.6			14.7	35.6	35.6
			0.34		16.3	33.1	33.1
				0.8	16.6	47.1	47.1
Means					**16.0 ± 0.88 A**	**39.9 ± 6.6 AB**	**39.9 ± 6.6 A**

[a]Within columns, the overall relative disease or culture absorbance means ± standard deviation shown in bold for co-culture, blend and pure strain treatments are significantly different if there are no 'capital' letters in common based on Student–Newman–Keuls pairwise comparison with significance criterion of $P < 0.05$.
[b]Within rows, the overall means for actual versus calculated relative disease are significantly different if there are no 'lower case' letters in common based on a paired *t*-test with significance criterion $P < 0.05$.
[c]Since relative disease did not vary significantly with treatment strain composition or treatment × pathogen concentration interactions, statistical one-way analysis of variance was conducted on the combined low and high level pathogen experiment data sets (0.5 or 1.5×10^6 Fusarium conidia/mL in 5 μL inoculations to Russet Burbank potato wounds). Fusarium dry rot lesions in controls without biocontrol agent had disease ratings averaging 10.5 or 13 mm, for low and high level disease challenge respectively, which corresponded to 100% relative disease.
[d]Viable cell concentrations of blend treatments were calculated from the pure stain plate counts reported since equal volume mixtures of pure strain cultures were prepared.
[e]Strain populations listed as 0 cells/mL were not present in the co-culture inocula, or they were not included in blends. The concentrations listed are prior to dilution 72 times for the wounded potato bioassay.

Table 2. Two-way analysis of variance of relative disease with strain composition of treatments and method of combining strains showed that significantly better Fusarium dry rot suppression was obtained by strain co-cultures than by similar strain mixtures created by blending pure strain cultures.

Treatment composition of strains				Least square mean of relative disease (%)[a,b]	
Strain presence (1) absence (0)				Strain combination method	
Pseudomonas fluorescens S11:P:12	*Pseudomonas fluorescens* S22:T:04	*Pseudomonas fluorescens* P22:Y:05	*Enterobacter cloacae* S11:T:07	Co-culture	Blend
1	1	1	1	28.2	25.4
1	1	1	0	39.8	30.8
1	0	1	1	39.8	50.5
1	1	0	1	30.7	34.3
0	1	1	1	22.5	45.5
1	0	1	0	25.0	44.8
1	1	0	0	26.7	30.0
1	0	0	1	36.8	32.2
0	1	1	0	23.5	48.0
0	0	1	1	27.1	40.6
0	1	0	1	33.1	71.9
Overall least square means $\pm$ standard error[b]				30.3 $\pm$ 2.4 A	41.3 $\pm$ 2.4 B
Significance				$P < 0.001$	

[a]All treatments were challenged with *Fusarium sambucinum* conidia at 0.5 or 1.5×10^6 per mL pipetted 5 μL per Russet Burbank potato wound. Dry rot lesions in controls without BCA had disease ratings averaging 10.5 or 13 mm, respectively pending pathogen inoculum size. Since relative disease did not vary significantly with the interaction of treatment × pathogen concentration, analysis was conducted on the combined low and high level pathogen data sets.

[b]The overall least square means of relative disease observed for strain co-cultures versus blends were significantly different ($P = 0.001$) based on Student–Newman–Keuls pairwise comparison method as designated by different letters. Relative disease did not vary significantly due to the strain composition of mixed strain treatments or the interaction of treatment strain composition × combination method.

blended strain combinations was $41.3 \pm 2.4\%$, while only $30.3 \pm 2.4\%$ for co-cultures, indicating that co-cultures reduced disease to a greater extent (69.7%) than had the blended strain combinations (58.7%). Using a $P < 0.05$ significance criterion, the variation of relative disease among the various strain compositions was not significant nor was the composition $\times$ combination method interaction.

An analysis was also done to determine whether biocontrol strain populations in co-cultures and blends were performing additively or synergistically. Co-culture refers to the technique of cultivating multiple strains together in one fermentation vessel starting from a low initial cell population density that multiplies and advances through growth phase to stationary phase. This process contrasts with blending large populations of strains together after they are grown separately as pure strain cultures from low initial cell concentration through growth phase to stationary phase. When strain populations are grown together prior to application, they may have the opportunity to benefit one another in such a way as to provide a final mixed population that suppresses disease more efficiently than a blend of pure cultures. On the other hand, co-cultured strains may not be compatible with one another, such that low population concentrations result or the end product does not suppress disease as well as observed for pure strains or a blend of pure strains. To test positive versus negative impact of strain combinations, the expected relative disease rating was calculated for comparison with the actual disease rating measured for each treatment comprised of multiple strains: $RDR_{calc} = (RDR_1 \times N_1 + RDR_2 \times N_2 + RDR_3 \times N_3 + RDR_4 \times N_4)/(N_1 + N_2 + N_3 + N_4)$, where N_1 is the concentration of strain population 1 (such as S11:P:12 viable cells/mL) in a multiple strain mixture, and RDR_1 is the relative disease rating observed for the pure strain population 1, and so forth for strains 2 through 4. Thus, RDR_{calc} for a mixed population is a population-weighted average of the observed disease ratings for single-strain treatments; or in other words, it is addition of the fractional performance contributions of each component strain. Comparison of RDR_{calc} with actual disease ratings of strain blends and co-cultures will indicate if the efficacy of strain components of a mixture are additive or if the method of combining them provides a synergistic advantage or disadvantage relative to the disease suppressiveness observed for pure strains applied individually.

After 72-h incubation both pure and co-cultures accumulated virtually the same biomass concentration as indicated by culture absorbances averaging 16.0 ± 0.88 and 15.9 ± 1, respectively (Table 1). In co-cultures containing *E. cloacae* strain S11:T:07, it was the dominant population in 72-h cultures, but low concentrations of other strains also existed. In co-cultures without *E. cloacae*, the 72-h populations were more equally divided among the *P. fluorescens* strains present. Nearly all co-cultures, regardless of the population distribution among strains, exhibited lower disease levels than expected by calculation of RDR_{calc} from the additive performance contributions of pure strains. The mean actual relative disease rating ($\pm$standard deviation) for co-cultures ($30.3 \pm 6.3\%$) was significantly lower than both the mean co-culture RDR_{calc} and the mean actual relative disease rating for blends, $42.9 \pm 5.3\%$ and $41.3 \pm 13.1\%$, respectively. In contrast, the mean actual relative disease rating for blends ($41.3 \pm 13.1\%$) was similar to that of pure strains ($39.9 \pm 6.6\%$) as well as the predicted mean value of RDR_{calc} for blends ($40.4 \pm 2.8\%$), obtained by adding the fractional efficacy contributions of the pure strains. These findings provide evidence that co-culturing strains stimulates

Table 3. Average biocontrol strain populations applied in treatments.

Treatment	Average population concentration $\times 10^{-8}$ (cells/mL)[a,b] S11:P:12	S22:T:04	P22:Y:05	Total
(A) Peoria laboratory wounded potato bioassays				
Co-culture	0.63 ± 0.15 B	1.49 ± 0.84 B	0.61 ± 0.55 B	2.72 ± 0.85 A
Blend	0.56 ± 0.34 B	0.84 ± 0.25 B	0.86 ± 0.26 B	2.26 ± 0.49 A
S11:P:12	1.61 ± 0.74 A	0	0	1.61 ± 0.74 A
S22:T:04	0	2.60 ± 1.55 A	0	2.60 ± 1.55 A
P22:Y:05	0	0	2.28 ± 1.19 A	2.28 ± 1.19 A
Control	0	0	0	0
	Average population concentration $\times 10^{-9}$ (cells/mL)[a,b]			
	S11:P:12	S22:T:04	P22:Y:05	Total
(B) Kimberly small pilot bioassays simulating commercial storage conditions				
Co-culture	2.22 ± 0.88 B	2.01 ± 2.8 B	4.52 ± 2.24 B	8.76 ± 3.50 B
Blend	1.48 ± 0.64 B	1.47 ± 1.40 B	2.39 ± 0.76 B	5.35 ± 2.72 BC
S11:P:12	3.65 ± 1.86 A	0	0	3.65 ± 1.86 C
S22:T:04	0	12.3 ± 1.5 A	0	12.3 ± 1.5 A
P22:Y:05	0	0	7.24 ± 5.70 A	7.24 ± 5.70 BC
Control	0	0	0	0

[a]Within columns, values not sharing a similar letter are significantly different based on Student–Newman–Keuls pairwise comparison method using $P < 0.05$ significance criterion.
[b]Standard deviations in mean values are indicated following the '±' symbol.

synergistic inter-strain activities that boost biocontrol efficacy. This synergy is apparently not developed in strains that are grown separately and mixed just prior to addition to potato wounds.

Consistency of co-culture, blend and pure strain biocontrol treatment efficacy across lab bioassays and small scale commercial storage simulation

In the investigation of the various strain combinations grown in baffled flasks, final treatment populations were not always equally distributed among the component strains in co-cultures where variable growth kinetics of strains occurred (Table 1). Similar populations in three strain mix fermentations after 72-h cultivation were achievable in fermentors by inoculating faster growing population P22:Y:05 to lower initial cell density than slower growing *P. fluorescens* strains S22:T:04 and S11:P:12 (Figure 1). The performance of this three-strain co-culture was compared with pure strains and the corresponding three-strain blend comprised of an equal volume mix of the three pure-strain populations grown under the same fermentor conditions. These treatments were compared in 16 experiments conducted in laboratory experiments in Peoria using wounded potato bioassays of dry rot or pink rot suppressiveness or in Kimberly, ID using small pilot simulations of commercial storage conditions during challenges of late blight, pink rot, and sprouting. The performance of treatments was then ranked within each experiment by calculating the dimensionless relative performance index of each treatment. Overall treatment

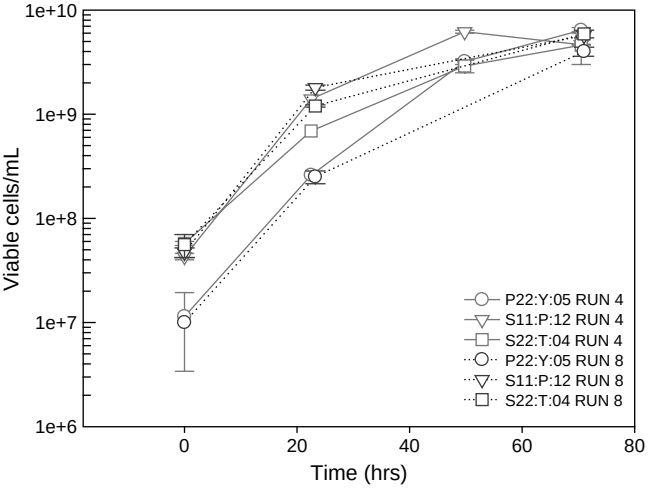

Figure 1. Growth of strains *P. fluorescens* strains S11:P:12, P22:Y:05, and S22:T:04 in duplicate co-culture fermentor runs.

performance could then be assessed and analyzed using analysis of variance and pairwise comparison techniques to determine superior treatments.

Table 3A,B shows the average strain populations in ready-to-apply treatments for the 16 laboratory and small pilot experiments as measured by dilution plating on selective media. These data show that co-culture, blend and pure strain treatments had similar total cell concentrations, and that blend and co-culture treatments had similar strain distributions. Table 4 summarizes the performance of the treatments in nine small-scale pilot tests in Kimberly, Idaho simulating commercial storage conditions. Table 5 summarizes the performance of similar treatments applied in seven laboratory experiments conducted in Peoria, IL using the wounded potato bioassay. Scanning these data, it is evident that at least one and usually more biocontrol treatments significantly reduced disease or sprouting relative to the control. Co-culture had a lower mean disease rating than the blend in 9 of 16 experiments. The co-culture led other treatments in incidence of successful significant disease or sprout reduction relative to the control: 14 of 16 attempts for co-culture, 11 of 16 attempts for blend, 10 of 13 attempts for S11:P:12, 8 of 13 attempts for S22:T:04, and 9 of 13 attempts for P22:Y:05.

For each bacterial and control treatment within an experiment, a relative performance index (RPI) was calculated, as listed in brackets to the right of means in Tables 4 and 5. RPI is a dimensionless value that is useful in combining data sets to use in overall ranking or statistical analysis of treatments submitted to various testing procedures. Given disease or sprout ratings normally distributed across the group of bacteria stains tested, the value of $F = (X - X_{avg})/s$ ranges from -2 to $+2$. Here, X designates a disease or sprout rating observed per treatment, and X_{avg} and s are the average and standard deviation, respectively, of all values observed for the group of bacteria treatments within a given experiment, such as the fall 2005 late blight bioassay at Kimberly. Since F decreases as disease or sprout suppressiveness

Table 4. Summary of treatment performance in Kimberly, Idaho small pilot tests simulating commercial storage.

	Late Blight (% surface coverage)[e,h]					Pink Rot (% surface coverage)[f,h]			Sprout weight (%)[h]
Treatment	Fall 2005	Winter 2006	Winter 2007	Fall 2007	Winter 2008	Winter 2007[g]	Winter 2008 RB	Winter 2008 RN	Fall 2005–April 2006
Co-culture	29.4[81] A	6.7[60] AB	6.3[84] A	12.1[68] A	2.50[51] A	50.8[54] AB	66.3[53] AB	90.8[69] A	0.438[80] A
Blend	45.3[49] B	5.8[74] A	11.2[40] AB	13.4[55] A	1.85[60] A	48.6[60] AB	59.3[73] A	89.3[80] A	0.603[51] AB
S11:P:12	-	-	6.9[78] A	15.4[34] A	1.63[63] A	40.9[81] A	75.8[26] BC	92.2[58] A	
S22:T:04	-	-	11.1[41] AB	11.9[70] A	1.39[66] A	53.8[46] B	59.8[72] A	95.1[37] AB	
P22:Y:05	-	-	10.5[47] AB	11.7[72] A	1.52[65] A	48.6[60] AB	60.8[69] A	92.8[54] A	
Control[d]	59.4[20] C	9.5[16] B	14.7[10] B	18.6[2] B	6.38[−5] B	70.6[0] C	82.5[7] C	99.8[2] B	0.788[19] B
Significance	$P<0.05$[a]	$P<0.075$[a]	$P<0.05$[a]	$P<0.1$[a]	$P<0.05$[a]	$P<0.05$[b]	$P<0.05$[b]	$P<0.1$[c]	$P<0.05$[a]

[a]Within the column, values having no letters in common are significantly different based on Student–Newman–Keuls pairwise comparison test.
[b]Within the column, values having no letters in common are significantly different based on Fisher's Protected LSD test using arcsine transformation if needed to normalize data.
[c]Values not sharing a similar letter are significantly different based on an unprotected LSD test.
[d]The control treatment consists of water instead of biocontrol agent applied to potatoes infested with pathogen.
[e]Late blight is incited by 4×10^4 sporangia/mL *Phytophthora infestans* sprayed at 1.6 mL per 8 oz Russet Burbank tuber.
[f]Pink rot is incited by 10^4–10^5 zoospores/mL, pending virulence, *Phytophthora erythroseptica* sprayed at 1.6 mL per 8 oz Russet Burbank (RB) or Russet Norkotah (RN) tubers.
[g]Data represent combined means of Russet Burbank and Russet Norkotah data sets since no significant treatment × cultivar interactions.
[h]Each number in brackets indicates the corresponding relative performance index (RPI) of the value relative to other data within the experiment column.

Table 5. Summary of treatment performance in Peoria laboratory wounded potato bioassays.

	Dry Rot disease rating (mm)[a,g]					Pink Rot disease rating (mm)[a,f,g]	
Treatment	Fall 2006[e]	Winter 2007[e2]	Spring 2007A[e]	Spring 2007B[e]	Fall 2007[e2]	Fall 2006	Winter 2007
Co-culture	5.3[63] A	5.2[71] A	23.0[62] A	28.7[77] A	6.7[62] A	54.5[39] AB	19.5[83] A
Blend	6.2[59] A	5.5[71] A	27.7[53] A	27.2[82] A	6.2[64] A	57.9[24] B	23.4[45] ABC
S11:P:12	4.5[63] A	9.3[64] A	27.8[52] A	35.0[53] AB	9.0[54] A	43.3[89] A	20.0[78] ABC
S22:T:04	3.2[65] A	19.3[46] A	16.3[75] A	35.3[52] AB	6.8[62] A	53.2[44] AB	26.9[11] C
P22:Y:05	7.2[57] A	17.8[49] A	23.3[61] A	44.1[19] AB	6.0[64] A	45.7[78] AB	23.0[49] ABC
Control[d]	37.6[−6] B	46.1[−1] B	56.3[−3] B	44.2[18] B	26.9[−5] B	57.7[25] B	24.6[33] BC
Significance	$P<0.001$[b]	$P<0.001$[b]	$P<0.001$[b]	$P<0.05$[b]	$P<0.001$[b]	$P<0.1$[c]	$P<0.1$[c]

[a]Disease ratings represent lesion width plus depth around a wound.
[b]Values not sharing a similar letter are significantly different based on Student–Newman–Keuls method pairwise comparison method.
[c]Values not sharing a similar letter are significantly different based on an LSD all pairwise comparisons test applied to the disease ratings.
[d]The control treatment consists of water + pathogen only applied to potatoes infested with pathogen.
[e]Dry rot is incited by 0.5×10^6 conidia *Fusarium sambucinum* per mL pipetted 5μ mL per wound on Russet Burbank potatoes.
[e2]Dry rot is incited by 1.5×10^6 conidia *Fusarium sambucinum* per mL pipetted 5μ mL per wound on Russet Burbank potatoes.
[f]Pink rot is incited by 1.5×10^4 zoospores *Phytophthora erythroseptica* pipetted 5μ mL per wound. Data represent combined means of Russet Burbank and Russet Norkotah data sets for each treatment.
[g]Each number in brackets indicates the corresponding relative performance index (RPI) of the value relative to other data within the experiment column.

improves, then RPI = (2 − F) × 100/4, such that the value of RPI ranges from ~0 to 100 percentile from least to most suppressive, respectively.

A one-way analysis of variance of RPI by treatment showed significant efficacy of biocontrol agents to suppress potato maladies relative to the control (Table 6). However, high standard deviation prevented statistical separation of the performances of biocontrol treatments although co-culture treatments performed most consistently across all 16 assays as indicated by exhibiting the highest overall mean RPI and the lowest relative standard deviation. A two-way analysis of variance in RPI with treatment and malady was performed which considered co-culture, blend and control across all 16 experiments conducted in Kimberly and Peoria. The result of this analysis is given in Table 7 and indicates that the overall mean RPI for the co-culture treatment was significantly higher than that for the blend.

Discussion

In past studies, we have demonstrated that cultivation conditions can have a major impact on cell yield and especially, biocontrol agent qualities – efficacy, shelf-life, and host compatibility. For example, our process for ranking dry rot antagonists relative to commercial potential involved growing the strains on three different liquid media of varying nutritional richness, and then applying them to potato wounds challenged by the pathogen. The rank of candidate strains based on the liquid culture growth and also disease suppressiveness of the product was found to vary widely with the nutritional environment provided during production of the biological control agent. These findings showed the critical importance of incorporating the liquid cultivation feature of industrial practice into the strategy used to choose a strain or strains for commercial development (Slininger et al. 1994; Schisler and Slininger 1997a; Schisler, Slininger, Hanson, and Loria 2000).

Prior research has shown that culture environment impacts metabolite accumulations and biocontrol agent quality. For example, managing the liquid culture pH and temperature during growth of *P. fluorescens* 2-79 improved its compatibility as a seed inoculant without sacrificing suppressiveness to wheat take-all. This was

Table 6. One-way analysis of variance by treatment shows significant efficacy of biocontrol agents to suppress potato maladies relative to the control, but high standard deviation prevents performance differentiation when interactions of treatment by potato malady type are ignored. Co-culture treatments perform most consistently across all 16 assays with lowest relative standard deviation.

Treatment	Mean RPI[a]		Standard deviation in mean RPI	Relative standard deviation (%)
Co-culture	65.9	A	±13.1	±19.8
Blend	58.7	A	±15.4	±26.2
S11:P:12	61.1	A	±18.1	±29.6
S22:T:04	52.9	A	±17.9	±33.8
P22:Y:05	57.2	A	±14.9	±26.0
Control	8.2	B	±12.2	±148.7

[a]Within a column, means not sharing a similar letter are significantly different based on the Student–Newman–Keuls pairwise comparison method ($P < 0.05$ significance criterion).

Table 7. Two-way analysis of variance of Relative Performance Index (RPI) with treatment × malady indicated that the relative biocontrol performance of the co-culture treatment was significantly better than that of the blend treatment over all 16 experiments conducted in Kimberly and Peoria.

Treatment	Potato malady bioassay	Average RPI treatment × malady[a]	Average RPI treatment groups[a,b]
Co-culture	Late Blight	68.7 ± 6.2	68.7 ± 4.4 A
	Pink Rot	59.6 ± 6.2	
	Dry Rot	66.5 ± 6.2	
	Sprouting	80.0 ± 13.9	
Blend	Late Blight	55.6 ± 6.2	57.2 ± 4.4 B
	Pink Rot	56.4 ± 6.2	
	Dry Rot	65.7 ± 6.2	
	Sprouting	51.2 ± 13.9	
Control	Late Blight	8.5 ± 6.2	10.3 ± 4.4 C
	Pink Rot	18.8 ± 6.2	
	Dry Rot	13.5 ± 6.2	
	Sprouting	0.5 ± 13.9	

[a]Least square means are reported, and the standard error in the means is given following each '±' symbol.
[b]Within the column, values having no letters in common are significantly different based on Student–Newman–Keuls pairwise comparison test using the $P < 0.075$ significance criterion.

accomplished by limiting the accumulation of its antifungal antibiotic phenazine-1-carboxylic acid, which also inhibits seed germination, without sacrificing suppressiveness to wheat take-all (Slininger and Shea-Wilbur 1995; Slininger et al. 1996b, 1997; Slininger, VanCauwenberge, Shea-Wilbur, and Bothast 1998). Culture environment is also likely to impact the metabolism and efficacy of our potato protective rhizobacteria which have been reported to produce at least one antifungal component per strain (Burkhead, Schisler, and Slininger 1995). The metabolite profile of *E. cloacae* strain S11:T:07, included in our co-culture study, has been studied in detail, and it is known to produce indoleacetic acid, phenyl acetic acid and tyrosol. These metabolites influence both disease suppression and sprouting (Burkhead, Slininger, and Schisler 1998; Slininger, Burkhead, and Schisler 2004).

In addition to influencing metabolite production, consequent efficacy and plant growth regulation, biocontrol agent cultivation conditions are also known to impact the shelf-life and efficacy of the product. For example, *P. fluorescens* 2-79 formulated from 24 to 48-h cultures had twice the drying survival rate but only half the storage life demonstrated by older stationary phase cells (72–96 h) (Slininger et al. 1996b, 1998) a feature that has also been observed in our potato protective rhizobacteria and that influenced our choice of a 72-h harvest for our co-cultures in the current research. The impact of cultivation conditions on biocontrol agent storage stability have been shown in virtually every other biocontrol system studied in our laboratory and not just those involving the control of plant diseases with rhizobacteria. Varying the carbon to nitrogen ratio and total carbon loading of a liquid medium for producing the bioherbicide *Colletotrichum truncatum* altered its dry storage stability as well as quantity, propagule type (conidia versus microsclerotia), and efficacy (Schisler, Jackson, and Bothast 1991; Jackson and Schisler 1995). Yield and desiccation-tolerance of blastospores of the mycoinsecticide *Paecilomyces fumosor-*

oseus required appropriate concentrations of amino acids (Jackson 1997; Jackson, McGuire, Lacey, and Wraight 1997). Carbon-to-nitrogen ratio and carbon loading were found to influence the freeze-drying survival and Fusarium headblight suppressiveness of *Cryptococcus nodaensis* OH 182.9 (Zhang, Schisler, Boehm, and Slininger 2005); and cold shock during liquid cultivation increased the storage shelf-life of this biocontrol agent after air drying (Zhang et al. 2006).

With the results reported here, we have now demonstrated that the unique fermentation environment fostered by multiple strains growing simultaneously also impacts biocontrol agent performance, and in the case of the strains reported, co-culturing has a positive overall impact on biocontrol strain performance in terms of both consistency and efficacy. As noted in our introductory section, improved consistency of biological control has been noted in previous reports describing applications of strains that have been mixed after cultivation. A draw back to this approach is that compared to a single large volume process, several smaller volume cultivations of pure strains represent higher registration and manufacturing costs. The results of the current research intensify the economic incentive to apply co-cultivation technology by showing that co-cultures outperform similar strain mixtures prepared as blends of strains produced in monoculture. Although the performance benefits of co-cultivations to biocontrol performance have not previously been documented, mixed Pseudomonad cultivations (with other *Pseudomonas* sp. or other genera) have been documented for many other applications including, for example: single cell protein production from bagasse pith (Rodriguez and Gallardo 1993); degradation of terephthalate in wastewater (Kimura and Ito 2001); production of poly(hydroxyalkanoate) blends from glycerol (Ashby, Solaiman, and Foglia 2005); the enhancement of oil degradation by co-culture of hydrocarbon degrading and biosurfactant producing bacteria (Kumar, Leon, De Sisto Materano, and Ilzins 2006); and many others. More recently, Wu, Ma, Wang, Yang, and Lou (2009) examined the synergistic growth of a salt tolerant *Pseudomonas fluorescens* Rs-198 with another bacterium Rs-5 in co-culture that may have potential for application in fertilizer preparation.

Our results showed that co-cultures of *P. fluorescens* strains could easily be designed to develop similar population densities even by simple adjustments in initial population densities to compensate for growth differences in strains (Figure 1). Although the *E. cloacae* most often dominated co-cultures which included it, the other co-inoculated *P. fluorescens* populations persisted at significantly lower levels and apparently synergized the performance of the final population in suppressing dry rot disease. Our results are not so surprising since *E. cloacae* is a facultative anaerobe, an attribute giving it a significant competitive advantage as dissolved oxygen is depleted in co-cultures with the obligate aerobe *P. fluorescens.* Entomoto (2003) reported that *Enterobacteriacea* grew competitively with *P. fluorescens* strains and prevented *Pseudomonas*-mediated spoilage of bean sprouts when seeds were soaking in water. Growth suppression of multi-species bacterial populations in batch cultures by a single dominant strain has been referred to in the literature as the Jameson Effect and often involves production of specific inhibitors by one species against another. Future work may reveal inoculum population management and nutritional or physical environment management techniques that would allow testing the biocontrol efficacy of more similar population distributions of *P. fluorescens* and

E. cloacae strains and allow investigation into the optimization of relative strain densities.

There are a number of avenues by which the unique environment fostered by co-cultivation may improve biocontrol performance, and it is likely that inter-strain communication mechanisms are involved. Gram negative bacteria partners have been reported to regulate anti-microbial metabolite production via a signaling system referred to as 'quorum sensing'. Quorum sensing (QS) is mediated by population size and the accumulation of acylated homoserine lactones (AHL) which stimulate the bacterial populations to express genes responsible for metabolite production (Wood and Pierson 1996). The local fermentation environment of the co-culture may synergize the impact of such signaling on subsequent biological control performance. Arrays of AHLs are known to be produced by many common rhizosphere bacteria, and they allow not only signaling between cells within a strain population, but also between cells of different strain populations (Pierson, Wood, Cannon, Blachere, and Pierson 1998). An AHL-mediated QS system, different from that noted for anti-microbial phenazine production, was reported to regulate cell surface properties (Zhang and Pierson 2001). Soil bacteria have also been shown to degrade AHLs, such as via lactonase activity (Molina et al. 2003), a feature suggesting the potential for curative biocontrol of bacterial diseases. In addition to metabolite regulation and disease suppression, QS has been implicated in many other aspects of biocontrol activity, including: regulation of biofilm formation and rhizosphere colonization (Wei and Zhang 2006); pathogen virulence, motility and fitness (Licciardello et al. 2007); indoleacetic acid (IAA) plant growth hormone synthesis (Müller et al. 2009), perhaps pertinent to IAA accumulation by our *E. cloacae* strain S11:T:07; and induced systemic resistance (Pang et al. 2009).

In addition to producing antifungal and sprout regulatory metabolites, we have recently identified the extracellular polysaccharide marginalan production by *P. fluorescens* S11:P:12 that not only improves its own survival during desiccation, but also that of P22:Y:05 and S22:T:04 (Slininger, Dunlap, and Schisler 2010). This feature suggests the community benefit of one strain for others grown in association with it. In previous research by others, exopolysaccharides (EPS) have been associated with improved desiccation tolerance in *Pseudomonas* sp. (Roberson and Firestone 1992) and have been implicated as triggers to induced systemic resistance in host plants. It has also been reported that sigma factor AlgU controls exopolysaccharide production and tolerance towards desiccation in *Pseudomonas fluorescens* CHA0 (Schnider-Keel, Lejbølle, Baehler, Haas, and Keel 2001). It is believed that the EPS matrix slows the rate of water loss within the microenvironment, which enables the microbe additional time to make the necessary metabolic adjustments needed for survival. Relating to the current research, the co-culture of exopolysaccharide-producing *Paenibacillus* sp. with a *Pseudomonas* species has been reported to extend the shelf-life of the *Pseudomonas* for potential biopesticide or biofertilizer use (Kozyrovska, Negrutska, Kovalchuk, and Voznyuk 2005). Thus, in addition to enhancing its biocontrol capacity, one of the benefits of our co-culture is that it includes an EPS-productive partner in *P. fluorescens* strain S11:P:12 that may protect bacteria against desiccation stress. The discovery of other mechanisms benefiting the function of the co-culture for consistent and efficacious biological control will likely be among the objectives of future research.

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